Atorvastatin Treatment Induced Peroxisome Proliferator-Activated Receptor α Expression and Decreased Plasma Nonesterified Fatty Acids and Liver Triglyceride in Fructose-Fed Rats

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ABSTRACT

We aimed to investigate the effect of atorvastatin (5 and 30 mg/kg/day for 2 weeks) on hepatic lipid metabolism in a well established model of dietary hypertriglyceridemia, the fructose-fed rat. Fructose feeding (10% fructose in drinking water for 2 weeks) induced hepatic lipogenesis and reduced peroxisome proliferator-activated receptor α (PPARα) expression and fatty acid oxidation. As a result, plasma and liver triglyceride and plasma apolipoprotein B (apoB) levels were increased. Atorvastatin, 5 and 30 mg/kg during 2 weeks, markedly reduced plasma triglyceride, but decreased apoB levels only at the highest dose tested (50%). Triglyceride biosynthetic enzymes and microsomal triglyceride transfer protein were unchanged, whereas liver PPARα, acyl-CoA oxidase, and carnitine palmitoyltransferase I mRNA levels (1.9-, 1.25-, and 3.4-fold, respectively) and hepatic fatty acid β-oxidation activity (1.25-fold) were increased by atorvastatin at 30 mg/kg. Furthermore, hepatic triglyceride content (45%) and plasma nonesterified fatty acids (NEFAs) (49%) were reduced. These results show for the first time that liver triglyceride increase in fructose-fed rats is linked to decreased expression of PPARα, which is prevented by atorvastatin treatment. The increase in PPARα expression caused by atorvastatin was associated with reduced liver triglyceride and plasma NEFA levels.

The assembly of apolipoprotein B (apoB)-containing lipoproteins requires both adequate supplies of lipids and functional microsomal triglyceride transfer protein (MTP). In the absence of either sufficient lipid or MTP activity, apoB is rapidly degraded with the subsequent blockage of lipoprotein assembly and secretion (Olofsson et al., 1999). Although the relative contribution of each lipid constituent of very low-density lipoprotein (VLDL) to the regulation of apoB secretion remains controversial, triglyceride synthesis seems to be essential (Benoist and Grand-Perret, 1996; Thompson et al., 1996).

There is increasing evidence that 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors lower plasma apoB concentrations by decreasing hepatic VLDL

secretion, although not all reports are consistent with this hypothesis (Thompson et al., 1996; Huff and Burnett, 1997). Furthermore, some compounds in this class, like atorvastatin, increase intracellular apoB degradation rates in HepG2 cells by mechanisms still unclear (Mohammadi et al., 1998). It has been proposed that HMG-CoA reductase inhibition may limit the availability of free cholesterol and/or cholesteryl ester for incorporation into VLDL (Krause and Newton, 1995).

The effect of HMG-CoA reductase inhibitors on cholesterol homeostasis is complex and may not be confined to the blockage of cholesterol biosynthesis (Goldstein and Brown, 1990). The decrease in cellular cholesterol content elicited by drug treatment leads to the activation of sterol regulatory element binding protein-2, enhancing the expression of genes involved in cholesterol synthesis and uptake. Nevertheless, treatment with colestipol and lovastatin also decreased the nuclear form of SREBP-1, which seems to be relatively selec-

tive in activating genes involved in fatty acid and triglyceride synthesis (Brown and Goldstein, 1997; Horton and Shimomura, 1999). Furthermore, the role of SREBPs in lipoprotein assembly has not been elucidated yet, although it has been reported that SREBP-1 and SREBP-2 bind to the MTP promoter (Sato et al., 1999).

On the other hand, HMG-CoA reductase inhibitors induce peroxisome proliferator-activated receptor α (PPARα) expression in endothelial cells (Inoue et al., 2000). PPARα regulates multiple enzymes and apolipoproteins implicated in lipid and lipoprotein metabolism (Keller et al., 2000). Thus, PPARα activation increases the hepatic fatty acid uptake, stimulates the conversion of fatty acids into acyl-CoA, and increases β-oxidation of fatty acids, which decreases the availability of fatty acids for triglyceride synthesis.

In the present study, we investigate the effect of atorvastatin treatment on MTP and key enzymes involved in hepatic triglyceride synthesis in an animal model of hypertriglyceridemia. Because adequate supply of free fatty acids is required for hepatic triglyceride synthesis, we also determined the effect of atorvastatin on key factors involved in hepatic fatty acid synthesis and oxidation, and the free fatty acid turnover in the adipose tissue.

Materials and Methods

Chemicals. 3-Hydroxy-3-methyl [3-^{14}C]glutaryl-coenzyme A, R,S-[2-^{14}C]mevalonic acid lactone, α-[^{32}P]dATP, and [^{14}C]palmitoyl-CoA were purchased from Amersham Biosciences (Freiburg, Germany), and [^{14}C]phosphatidic acid was from PerkinElmer Life Sciences (Boston, MA). Reverse transcription-polymerase chain reaction (RT-PCR) buffers and reagents were from Invitrogen (Paisley, UK), except hexanucleotide mix and random priming mix purchased from Boehringer Ingelheim GmbH (Heidelberg, Germany). Anti-apolipoprotein B, human (rabbit) was from Calbiochem-Novabiochem (Darmstadt, Germany) and horseradish peroxidase-conjugated anti-rabbit IgG from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were of analytical grade and were obtained from Sigma-Aldrich (St. Louis, MO). Atorvastatin calcium was supplied by Pfizer S.A. (Madrid, Spain).

Animals and Experimental Design. Male Sprague-Dawley rats (Harlan, Gannat, France) were maintained with water and food ad libitum at constant humidity and temperature with a light/dark cycle of 12 h. After 5 days, the animals (average weight 185 ± 9) were randomized into four groups: a control group, a fructose-supplemented group (fructose), and two groups supplemented with fructose and treated with atorvastatin at 5 or 30 mg/kg (ATV5 and ATV30, respectively). Fructose was supplied as 10% solution in drinking water for 2 weeks. Control animals received no supplementary sugar. Previous studies had shown that such rats were hypertriglyceridemic, normoglycemic, normoinsulinemic, and nonobese (Park et al., 1997). Rats consumed the same amount of fructose regardless of the treatment and no significant differences in body weight or daily intake of fructose were observed between treatment groups. Atorvastatin was administered by daily oral gavage for 2 weeks, using an aqueous carboxymethylcellulose suspension vehicle (0.5% carboxymethylcellulose plus 0.1% Tween 80 in water). Drug doses were given daily at 3:00 to 4:00 PM. Animals were killed by decapitation under diethyl ether anesthesia between 9:00 and 10:00 AM. All procedures were conducted in accordance with the principles and guidelines established by the University of Barcelona Bioethics Committee, as stated in Law 5/1995 (July 21, from the Generalitat de Catalunya).

Sample Preparation. Rat livers were excised, perfused, and homogenized in a buffer composed of 150 mM NaCl, 1 mM dithiothreitol, 30 mM EDTA, and 50 mM KH$_2$PO$_4$, pH 7.4. The subcellular

fractions were obtained by differential centrifugation and stored at $-80°C$ until needed. The protein concentration of each fraction was determined by the method of Bradford (1976). Liver and epididymal white adipose tissue (10–100 mg) of each rat was immediately frozen in liquid N$_2$ and used for the extraction of total RNA with the Ultraspec (Biotecx Laboratories, Houston, TX) reagent, in accordance with the manufacturer's guidelines. Blood samples were collected at the time of death in heparinized tubes; plasma was obtained by centrifugation and stored at $-80°C$ until needed.

Lipid, Glucose, Insulin, and apoB Analysis. Plasma total cholesterol and triglyceride concentrations were measured with the Roche Applied Science (Mannheim, Germany) colorimetric tests (Monotest Cholesterol CHODPAP 290319 and Peridochrom Triglyceride GPO-PAP 701882). VLDL and low-density lipoprotein from plasma samples were precipitated by using reagent 543004, also from Roche Applied Science, and high-density lipoprotein (HDL)-cholesterol concentration was determined in the supernatant.

Liver lipid was extracted and measured as described previously, using the homogenate fraction (Verd et al., 1999). Free cholesterol and triglyceride concentrations were determined as described above.

Plasma insulin and glucose concentration were determined with Rat Insulin RIA kit (LinCo Research, St. Charles, MO) and colorimetric test (glucose test no. 115A; Sigma-Aldrich), respectively.

Rat plasma apoB was measured by immunoblotting (Calvo and Enrich, 2000). Plasma protein (0.5 μg) was applied to 7% SDS-polyacrylamide gel electrophoresis and transferred to Immobilon-P transfer membranes (Millipore Corporation, Bedford, MA). ApoB was detected using a rabbit polyclonal antibody raised against human apoB (1:1000 dilution) and a horseradish peroxidase-conjugated anti-rabbit IgG (1:4000 dilution). Detection was performed with the ECL kit (Amersham Biosciences), the bands were quantified by image analysis (Vilbert Lourmat Imaging, Scientific and Technical Services, University of Barcelona, Barcelona, Spain), and apoB levels were expressed in arbitrary units.

Enzyme Assays. HMG-CoA reductase, phosphatidate phosphohydrolase (PAP), and hepatic fatty acid β-oxidation activities were determined as described previously (Lazarow, 1981; Alegret et al., 1998; Verd et al., 1999), using as source of enzyme liver microsomes, postmitochondrial or homogenate fraction, respectively. MTP activity was assayed by the commercial kit WAK-MTP-100 (WAK-Chemie Medical, Bad Homburg, Germany), in accordance with the manufacturer's guidelines.

mRNA Analysis. The relative levels of specific mRNAs were assessed by RT-PCR, basically as described previously (Cabrero et al., 1999). Single-stranded cDNA was synthesized from 1 μg (liver) or 0.5 μg (adipose tissue) of total RNA using 125 ng of random primers and 200 U of M-MLV-reverse transcriptase in a buffer containing 50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl$_2$, 10 mM dithiothreitol, 20 U of RNaseOut, and 500 μM of each dNTP in a total volume of 20 μl. The reverse transcription reaction was performed for 60 min at 37°C. PCR was carried out using a 5-μl aliquot of the reverse transcriptase reaction mix, 0.5 μg of both sense and antisense primers, 200 μM dNTPs, 1 U of TaqDNA polymerase, and 0.25 μCi of α-[^{32}P]dATP in 20 mM Tris-HCl, pH 8.5, and 2.5 mM Mg Cl$_2$ (final volume 50 μl). To avoid unspecific annealing, cDNA and Taq polymerase were separated from primers and dNTPs by using a paraffin plug. During the initial denaturation step, paraffin melts (at 60°C) and allows all the reaction components to mix. PCR was performed in a thermocycler (MJ Research, Watertown, MA) equipped with a Peltier system and temperature probe. After the denaturation of primers and cDNA at 94°C for 1 min, the cycling program was performed as follows: 92°C for 1 min, 60°C (63°C for SREBP-1C) for 1 min and 15 s, and 72°C for 1 min and 50 s. In the last cycle, a final 5-min extension step at 72°C was performed. To confirm the absence of contamination, negative controls were included in each experiment. Preliminary experiments had been carried out to establish the conditions for exponential amplification of all the genes studied, by calculating the range of cycle number at which a linear relationship is detected between

TABLE 1
Primers used for the PCR reaction

Primers	Sense and Antisense	PCR Product bp	GenBank Accession No.
ACC	5'-CGAGGCCGCTCAGCAACAGTA-3' 5'-TGGGTTCCTCGGAGGCTTCTC-3'	211	AB004329
ACO	5'-ACTATATTTGGCCAATTTTGTG-3' 5'-TGTGGCAGTGGTTTCCAAGCC-3'	196	NM017340
APRT	5'-AGCTTCCGGACTTCCCCATC-3' 5'-GACCACTTTCTGCCCCGGTTC-3'	329	L04970
ASP	5'-AAACCAGCCCCTGAGACAGCC-3' 5'-GCACAGCGGCACATTTCATTG-3'	440	M29866
FAS	5'-GTCTGCAGCTACCCACCCGTG-3' 5'-CTTCTCCAGGGTGGGGACCAG-3'	214	M76767
FAT/CD36	5'-ATTGGCACAGATGCAGCCTCC-3' 5'-TGCCAATGTCCAGCACACCAT-3'	256	AF111268
HMG-CoA Rd	5'-CCGACAAGAAACCTGCTGCCA-3' 5'-CAGTGCCACACACAATTCGGG-3'	470	X55286
HSL	5'-CCTGAGGCCTTTGAGATGCCA-3' 5'-AGGGACACGGTGATGCAGAGG-3'	491	X51415
L-CPT-1	5'-TATGTGAGGATGCTGCTT-3' 5'-CTCGGAGAGCTAAGCTTG-3'	629	AF029875
LPL	5'-CCCTGGGGAGTTTGGCTCCAGA-3' 5'-CCCAGCTGGATCCAAGCCAGT-3'	580	L03294
SREBP-1c	5'-TCACAGATCCAGCAGGTCCCC-3' 5'-GGTCCCTCCACTCACCAGGGT-3'	180	L16995
SREBP-2	5'-CATGGACACCCTCACGGAGCTGGGCGACGA-3' 5'-TGCATCATCCAATAGAGGGCTTCCTGCTC-3'	920	U12330
PPARα	5'-GGCTCGGAGGGCTCTGTCATC-3' 5'-ACATGCACTGGCAGCAGTGGA-3'	654	M88592
PPARγ	5'-TGGGGATGTCTCACAATGCCA-3' 5'-TTCCTGTCAAGATCGCCCTCG-3'	200	AB011365

bp, base pair(s).

input RNA and final product. For each primer set, an increasing number of PCR cycles with otherwise fixed conditions was performed to determine the optimal number of cycles. The same procedure was followed for RNA concentration (Gause and Adamovicz, 1995). Adenosyl phosphoribosyl transferase (APRT) was used as internal control and coamplified with target sequences in the same tube, except for SREBP-2, fatty acid synthase (FAS), acyl-CoA oxidase (ACO), and lipoprotein lipase (LPL). These sequences were amplified in parallel with APRT in separate tubes and in duplicate. The number of cycles was 30 for SREBP-2; 22 for HMG-CoA reductase and PPARγ; 25 for PPARα; 24 for ACO; 23 for acetyl-CoA carboxylase (ACC), hormone-sensitive lipase (HSL), SREBP-1c, and L-CPT-1; 18 for FAS and LPL; and 20 for fatty acid translocase (FAT/CD36). Twenty-three cycles were used for APRT when it was amplified separately. Primer sequences and resulting PCR products are listed in Table 1. Five microliters of each PCR reaction mixture was subjected to electrophoresis in 5% polyacrylamide gel in 1× Tris borate-EDTA. Gels were dried, autoradiographed on RX-OMAT S Kodak film, and quantified by image analysis (Vilbert Lourmat Imaging). The mRNA levels were always expressed as ratio to APRT mRNA levels.

Statistics. The results are the mean ± standard error mean of n experiments assayed in duplicate. Significant differences were established by a one-way analysis of variance followed by the Student-Newman-Keuls multiple comparison test, using the computer program GraphPad InStat, version 2.03 (GraphPad Software, San Diego, CA). Logarithms were used to calculate statistics when the variance was not homogeneous. The level of statistical analysis was set at $P < 0.05$.

Results

Lipid, Glucose, Insulin, and apoB Levels. Hypertriglyceridemia was induced in rats by fructose feeding, with plasma triglycerides and apoB level values higher than in control animals (by 2.12- and 1.85-fold, respectively) (Table 2). The increase in plasma triglyceride levels was not associated with an insulin-resistant state because NEFA and insulin (Fig. 1), as well as glucose plasma concentrations (153 ± 6 and 160 ± 9, for control and fructose group, respectively), were not modified by fructose feeding. Plasma and liver cholesterol levels were not affected by fructose feeding.

Atorvastatin did not lower plasma (Table 2) or liver cholesterol content (0.98 ± 0.04, 0.75 ± 0.04, 0.84 ± 0.07, and 0.87 ± 0.07 mg of cholesterol/mg of liver homogenate protein for control, fructose, ATV5, and ATV30 treatment groups,

TABLE 2
Effect of atorvastatin treatment on plasma lipid and apoB levels in fructose-fed rats
Data are the mean ± S.E.M. (n = 6). Rats received no supplementary fructose (control), wer fed fructose without treatment (fructose), or were fed fructose and treated with 5 or 30 mg/kg atorvastatin (ATV5 or ATV30)

	Control	Fructose	ATV5	ATV30
Total cholesterol (mg/dl)	110 ± 7	111 ± 6	110 ± 10	108 ± 6
HDL-cholesterol (mg/dl)	74 ± 4	76 ± 5	71 ± 8	75 ± 5
Triglyceride (mg/dl)	99 ± 9	210 ± 36[a]	133 ± 10[b]	100 ± 9[b]
apoB (arbitrary units)	0.826 ± 0.124	1.530 ± 0.128[a]	1.226 ± 0.218	0.774 ± 0.100[b]

[a] Values significantly different from control.
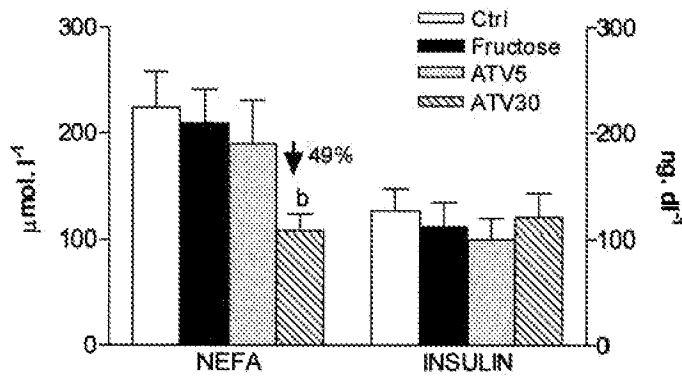[b] Values significantly different from fructose ($P < 0.05$).

Fig. 1. Effect of atorvastatin treatment on plasma NEFAs and insulin levels in fructose-fed rats. Data are the mean ± S.E.M. (n = 6). Plasma NEFA was expressed as micromoles per liter of plasma and insulin levels as nanograms per deciliter of plasma. Rats received no supplemental fructose (ctrl), were fed fructose without treatment (fructose), or were fed fructose and treated with 5 or 30 mg/kg atorvastatin (ATV5 and AVT30, respectively). b, values significantly different from fructose (P < 0.05).

respectively) in this animal model of hypertriglyceridemia. Nevertheless, it reduced the plasma triglyceride levels of the fructose group by 37 and 52%, at the 5- and 30-mg/kg doses, respectively (Table 2). Liver triglyceride content and plasma apoB levels were significantly reduced only by the highest dose of atorvastatin (45 and 50% versus fructose group, respectively; Fig. 3; Table 2). Plasma NEFA was also markedly decreased by atorvastatin at the 30-mg/kg dose (49% versus fructose group), whereas insulin (Fig. 1) and glucose levels were unchanged (153 ± 6, 160 ± 9, 155 ± 7, and 157 ± 8 mg/dl for control, fructose, ATV5, and ATV30 treatment groups, respectively).

Hepatic Lipid Synthesis and VLDL Assembly. mRNA levels of SREBP-2 and HMG-CoA reductase were unchanged by fructose feeding, but significantly enhanced by atorvastatin treatment, as well as HMG-CoA reductase activity (2.6-, 2.8-, and 5.6-fold, versus fructose group, respectively, for 30 mg/kg atorvastatin; Table 3). Despite that statins are competitive inhibitors of HMG-CoA reductase, when microsomal HMG-CoA reductase activity from livers of statin-treated animals is measured, an increase in enzyme activity is detected because the inhibitors have been removed from the microsomes during sample obtention.

Fructose feeding enhanced PAP activity and the mRNA levels of FAS (1.79- and 5.1-fold versus control group, respectively), but neither the PAP activity nor the mRNA level of the fatty acid biosynthetic enzymes was affected by atorva-

statin. The mRNA levels of SREBP-1 and the MTP activity were not affected by fructose feeding or atorvastatin treatment (Table 3).

PPARα and Fatty Acid Oxidation. Fructose feeding decreased the hepatic capacity for oxidation of fatty acids by 24%, regarding control values (Fig. 2). Furthermore, the expression of acyl-CoA oxidase and liver-carnitine palmitoyl transferase I, the rate-limiting enzymes controlling peroxisomal and mitochondrial fatty acid oxidation, were reduced by fructose feeding (54 and 84%, respectively; Fig. 2). Because the genes coding for both enzymes are controlled by PPARα, we determined further the effect of fructose feeding on the hepatic expression of this nuclear receptor. Indeed, fructose-fed rats showed a 57% decrease in the expression of PPARα with respect to control animals (Fig. 3).

Atorvastatin treatment dose dependently reverted the reduced expression of liver PPARα produced by the fructose-supplemented diet (Fig. 3). Atorvastatin (5 mg/kg) did not significantly change the expression of PPARα, ACO, and CPT-I, although a trend toward increased values with respect to those presented by the fructose animals is observed. At the highest dose tested (30 mg/kg), atorvastatin practically normalized the expression of PPARα, and consequently, increased the expression of ACO (although in this case, the change was not statistically significant) and CPT-I (Fig. 2). The final result was an almost normalization of the hepatic fatty acid oxidation activity, compared with values obtained in control animals (Fig. 2).

Free Fatty Acid Turnover in Adipose Tissue. Fructose feeding did not modify the mRNA levels of the key regulators of NEFA delivery into the circulation, LPL or HSL (Table 4). Factors stimulating uptake and tissue retention of fatty acids such as PPARγ, FAT/CD36, and ASP were also unaffected by fructose feeding (Table 4).

Treatment with 30 mg/kg atorvastatin significantly decreased the mRNA levels of the ASP precursor, with a concomitant increase in the mRNA levels of HSL (42 and 32% versus fructose group, respectively; Table 4). The mRNA levels of PPARγ, LPL, and FAT/CD36 were not affected by atorvastatin administration.

Discussion

Fructose feeding provides a dietary model of hypertriglyceridemia because fructose stimulates hepatic de novo lipogenesis and VLDL production (Kazumi et al., 1986) and

TABLE 3

Effect of atorvastatin treatment on key factors involved in hepatic lipid synthesis and VLDL assembly

Data are the mean ± S.E.M. (n = 6). MTP activity is expressed in fluorescence arbitrary units (a.u.)/h/mg. The mRNA levels are expressed as ratio to the reference gene APRT. Rats received no supplemental fructose (control), were fed fructose without treatment (fructose), or were fed fructose and treated with 5 or 30 mg/kg atorvastatin (ATV5 or ATV30), respectively.

	Control	Fructose	ATV5	ATV30
HMG-CoA Rd activity (pmol/min/mg)	0.044 ± 0.009	0.063 ± 0.016	0.149 ± 0.031[b]	0.355 ± 0.05[b,c]
PAP activity (nmol/min/mg)	0.455 ± 0.021	0.813 ± 0.075[a]	1.002 ± 0.046	0.887 ± 0.034
MTP activity (arbitrary units/h/mg)	186 ± 29	222 ± 17	234 ± 20	186 ± 19
HMG-CoA Rd mRNA (arbitrary units)	1.170 ± 0.14	0.797 ± 0.09	1.370 ± 0.19[b]	2.250 ± 0.39[b,c]
SREBP-1c mRNA (arbitrary units)	0.682 ± 0.13	0.595 ± 0.23	0.440 ± 0.19	0.572 ± 0.09
SREBP-2 mRNA (arbitrary units)	0.366 ± 0.08	0.239 ± 0.06	0.819 ± 0.09[b]	0.622 ± 0.09[b]
FAS mRNA (arbitrary units)	0.23 ± 0.04	1.20 ± 0.21[a]		1.01 ± 0.20
ACC mRNA (arbitrary units)	1.79 ± 0.19	1.99 ± 0.34		1.80 ± 0.21

[a] Values significantly different from control (P < 0.001).
[b] Values significantly different from fructose (P < 0.05).
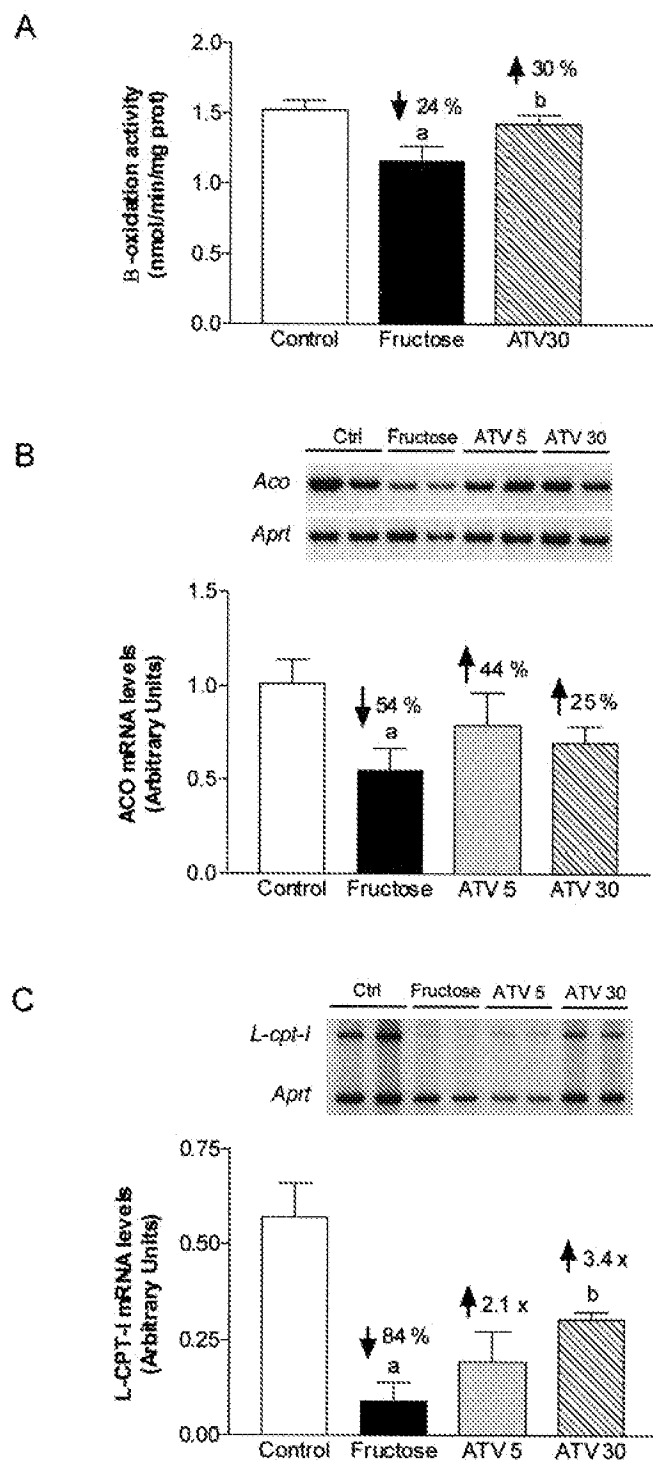[c] Values significantly different from ATV5 (P < 0.01).

A

B

C

Fig. 2. Changes in hepatic fatty acid oxidation (A) and APRT-normalized mRNA levels, as measured by RT-PCR, for mRNA levels of ACO (B) and L-CPT-I (C) in fructose-fed rats with and without atorvastatin treatment. For ACO and L-CPT-I, a representative autoradiography of the signals for specific and APRT mRNAs from two animals of each treatment group is shown. Data are the mean ± S.E.M. (n = 6). Rats received no supplemental fructose (control), were fed fructose without treatment (fructose), or were fed fructose and treated with 30 mg/kg atorvastatin (ATV30). a, values significantly different from control; b, values significantly different from fructose (P < 0.05).

impairs triglyceride removal (Hirano et al., 1989). Rodents fed chronically with diets containing high percentages of fructose (60–66%) develop insulin resistance (Taghibiglou et
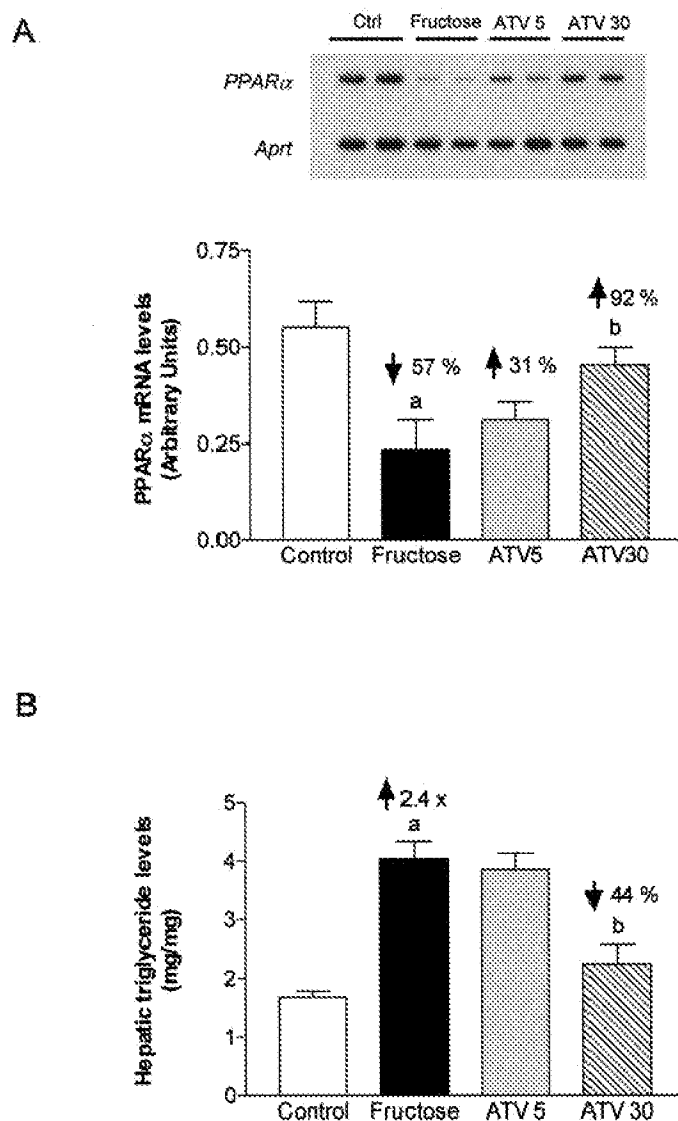
A

B

Fig. 3. Effect of atorvastatin treatment on liver PPARα mRNA and hepatic triglyceride content. A, APRT-normalized mRNA levels for PPARα, as measured by RT-PCR. A representative autoradiography of the signals for specific and APRT mRNAs from two animals of each treatment group is shown. B, hepatic triglyceride levels. Data are the mean ± S.E.M. (n = 6). Rats received no supplementary fructose (control), were fed fructose without treatment (fructose), or were fed fructose and treated with 5 or 30 mg/kg atorvastatin (ATV5 and ATV30, respectively). a, values significantly different from control; b, values significantly different from fructose (P < 0.05).

al., 2000). In the present study, 10% fructose feeding leads to hypertriglyceridemia without inducing an insulin-resistant state, in accordance with Park et al. (1997). Accordingly, plasma triglyceride and apoB levels were moderately increased, whereas plasma insulin, glucose, and NEFA levels were unchanged. FAS mRNA levels, PAP activity, and hepatic triglyceride content were also increased by fructose feeding, although the hepatic expression of SREBP-1, a key transcription factor controlling fatty acid and triglyceride biosynthesis, was not changed.

HMG-CoA reductase inhibitors deplete cellular cholesterol, causing SREBPs activation and enhancing the expression of target genes such as HMG-CoA reductase and SREBP-2 (Brown and Goldstein, 1997). Generally, despite the induction of HMG-CoA reductase, HMG-CoA reductase

TABLE 4
Effect of atorvastatin treatment on the mRNA levels of key factors involved in free fatty acid turnover in the adipose tissue.
Data are the mean ± S.E.M. ($n = 6$). The mRNA levels are expressed as ratio to the reference gene APRT. Rats received no supplemental fructose (control), were fed fructose without treatment (fructose), or were fed fructose and treated with 5 or 30 mg/kg atorvastatin (ATV5 or ATV30).

	Control	Fructose	ATV5	ATV30
ASP (arbitrary units)	0.964 ± 0.064	0.829 ± 0.10	0.848 ± 0.10	0.485 ± 0.08^a
PPARγ (arbitrary units)	7.57 ± 1.47	7.07 ± 1.72	7.60 ± 1.64	7.57 ± 0.87
FAT/CD36 (arbitrary units)	1.69 ± 0.11	2.23 ± 0.36	1.82 ± 0.30	2.03 ± 0.25
HSL (arbitrary units)	2.65 ± 0.17	2.73 ± 0.27	2.67 ± 0.30	3.61 ± 0.23^a
LPL (arbitrary units)	1.23 ± 0.08	1.11 ± 0.12	1.22 ± 0.13	1.43 ± 0.11

^{a} Values significantly different from fructose ($F < 0.05$).

activity and cholesterol synthesis remain inhibited while statins are present inside the hepatocyte and plasma cholesterol levels decrease. However, it is well established that HMG-CoA reductase inhibitors lower plasma triglyceride rather than cholesterol in rats because HDL is the main transporter of rat plasma cholesterol (Krause and Newton, 1995). Accordingly, atorvastatin treatment increased SREBP-2 mRNA levels and HMG-CoA reductase mRNA levels and activity. Plasma cholesterol levels remained unchanged, whereas triglyceride levels were markedly reduced in fructose-fed rats treated with 5 or 30 mg/kg atorvastatin.

The mechanisms underlying the triglyceride-lowering effect of HMG-CoA reductase inhibitors are not fully elucidated (Huff and Burnett, 1997; Mohammadi et al., 1998; Schoonjans et al., 1999; Sniderman et al., 2000). Because triglyceride synthesis and MTP expression are the major factors in posttranslational regulation of apoB secretion (Lewis, 1997) and both seem to be regulated by SREBPs, we studied the effect of HMG-CoA reductase inhibitors on MTP and the key enzymes involved in fatty acid and triglyceride synthesis. Neither fatty acid biosynthetic enzyme mRNA nor PAP nor MTP activities were affected by drug treatment, although plasma apoB levels were reduced by 30 mg/kg atorvastatin (Table 2). Recently, Burnett et al. (1999) have suggested that the magnitude of decrease in hepatic VLDL apoB secretion caused by statin treatment was determined by the extent of HMG-CoA reductase inhibition. Previous results from our group indicate that atorvastatin reduces plasma triglycerides in a rabbit model of hypertriglyceridemia without affecting ACAT activity or liver cholesteryl esters content (Verd et al., 1999). Thus, taken together, our results suggest that the HMG-CoA reductase inhibition caused by treatment with 5 mg/kg atorvastatin is sufficient to decrease cholesterol availability, limiting the amount of triglyceride assembled into nascent VLDL, as suggested also by Krause and Newton (1995). However, higher doses of atorvastatin are necessary to block apoB secretion and lower plasma apoB levels.

On the other hand, treatment with 30 mg/kg atorvastatin had other effects that may contribute to the triglyceride-lowering effect. Thus, both plasma NEFA levels and hepatic triglyceride content were markedly reduced by 30 mg/kg atorvastatin. Accordingly, we studied the effect of atorvastatin on key factors controlling fatty acid availability for triglyceride synthesis.

Fatty acids for triglyceride synthesis can be derived from plasma free fatty acids. The supply of plasma free fatty acids to the liver depends on the release of FFA by 1) lipolysis of circulating lipoproteins, 2) the rate of FFA release from adipose tissue, and 3) the rate at which these fatty acids are taken up and reesterified by tissues (Fig. 4). Therefore, we studied the effect of atorvastatin on the main regulatory
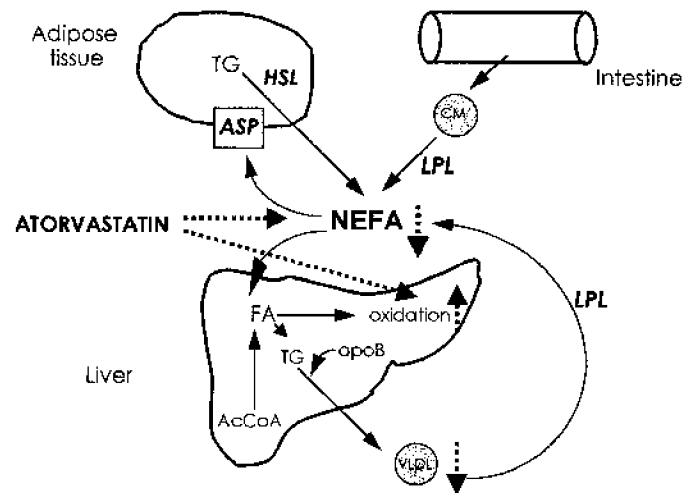

Fig. 4. Regulation of NEFA metabolism. The effect of atorvastatin treatment is represented with dotted lines. AcCoA, acetyl-coenzyme A; CM, chylomicrons; FA, fatty acid; TG, triglycerides.

factors involved in each one of these processes: LPL (Jansen et al., 1998), HSL (Frayn, 1998), and PPARγ (Keller et al., 2000); ASP (Sniderman et al., 1998); and FAT/CD36 (Coburn et al., 2000), respectively. Our data show that treatment with 30 mg/kg atorvastatin increased the adipose tissue mRNA levels of HSL and reduced ASP precursor mRNA levels. The data obtained seem to be contradictory because both the decrease in ASP precursor mRNA and the increase in HSL mRNA levels should theoretically increase plasma NEFA levels. Thus, these changes might be considered as a homeostatic response of adipocytes to decreased plasma NEFA levels rather than a direct effect of atorvastatin on this pathway.

On the other hand, fatty acid availability to triglyceride synthesis not only depends on plasma free fatty acid supply to the liver but also on de novo fatty acid synthesis and oxidation. It is well known that hepatic mitochondrial and peroxisomal fatty acid oxidation is regulated by the nuclear receptor PPARα (Keller et al., 2000). In the present study, we demonstrate for the first time that fructose feeding induces a down-regulation of hepatic PPARα expression. As a consequence, the expression of the genes coding for the two rate-limiting enzymes of the peroxisomal and mitochondrial fatty acid oxidation, acyl-CoA oxidase and carnitine palmitoyl-CoA transferase I, known to be regulated by PPARα (Keller et al., 2000; Louet et al., 2001), is markedly reduced, together with the liver fatty acid oxidation activity. These results suggest that the mechanism involved in the lipid metabolic disturbance produced by fructose feeding might be similar to pathological conditions in humans, increasing the usefulness of

this model for the investigation of potential therapeutic drugs. What we do not know is what are the molecular events relating fructose feeding to PPARα down-regulation. Although it has been reported that insulin treatment of primary hepatocytes decreases the expression of the PPARα gene (Sugden et al., 2001), our fructose-fed animals had practically identical plasma insulin concentrations to controls, discarding an insulin effect on PPARα expression. Nevertheless, it is interesting to note that a very recent report indicates that high concentrations of glucose are also able of down-regulate the expression of PPARα and that of PPARα target genes, such as acyl-CoA oxidase, in pancreatic β-cells (Roduit et al., 2000).

Activation of PPARα with PPARα agonists, such as fibrates, not only reduces plasma triglycerides but also greatly decreases fatty liver in humans (Chitturi and Farrell, 2001). Conversely, the partial or total ablation of PPARα transcriptional activity has been involved in the accumulation of hepatic triglyceride and the appearance of fatty liver (i.e., alcoholic steatosis) (Kersten et al., 1999; Galli et al., 2001). Our results show that the treatment with atorvastatin increased dose dependently hepatic PPARα mRNA levels. As a consequence, the expression of acyl-CoA oxidase and CPT-I was increased and the hepatic fatty acid oxidation activity was almost restored at the highest dose of atorvastatin. These effects were associated with the decrease (44%) in the hepatic triglyceride content (Fig. 3). Inoue et al. (2000) have previously shown that HMG-CoA reductase inhibitors induce PPARα expression in cultured endothelial cells; our work confirms that HMG-CoA reductase inhibitors are able to induce PPARα expression and activity in the liver of living animals. Very recently, it has been demonstrated that inhibition of the Rho-signaling pathway after incubation of cultured cells with statins reduces the phosphorylation state of PPARα and increases its transcriptional activity (Martin et al., 2001). More work is needed to see whether such an effect in vivo is able to increase not only the activity but also the transcription of the *ppara* gene itself.

Furthermore, it is well known that PPARα agonists decrease plasma NEFA levels (Shepherd et al., 1991; Catapano, 1992). In the present study, atorvastatin induces PPARα expression and fatty acid oxidation, which might lead to a decrease in fatty acid availability for triglyceride synthesis. Nevertheless, the enhanced hepatic mitochondrial fatty acid oxidation caused by atorvastatin treatment may not suffice to account for the plasma NEFA reduction. We hypothesize that atorvastatin might also induce PPARα expression in other tissues, such as muscle. In this case, the addition of effects caused by the induction of PPAR-target genes in liver and muscle could be sufficient to increase the NEFA flux to these tissues and reduce plasma NEFA levels. Therefore, the induction of PPARα expression produced by atorvastatin treatment could be also related with the reduction of plasma NEFA levels, although we cannot discard that other factors may be involved. If the NEFA-lowering effect of atorvastatin was confirmed in clinical studies, atorvastatin could be a useful tool in the treatment of a variety of chronic metabolic diseases because elevation of plasma NEFA concentration has been suggested to lie at the heart of the insulin resistance syndrome and its associated dyslipidemia.

In conclusion, for the first time, we have shown that hepatic triglyceride accumulation induced by fructose feeding is probably associated to a down-regulation of PPARα. This effect is prevented by atorvastatin treatment that increases PPARα expression and fatty acid oxidation and reduces plasma NEFA levels (Fig. 4). Our results suggest that with high doses of statins and/or very potent statin molecules, such as atorvastatin, triglyceride reduction might result not only from limited cholesterol availability but also from the cross talk with PPARα, a nuclear receptor whose activity is determinant in the control of fatty acid oxidation.

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